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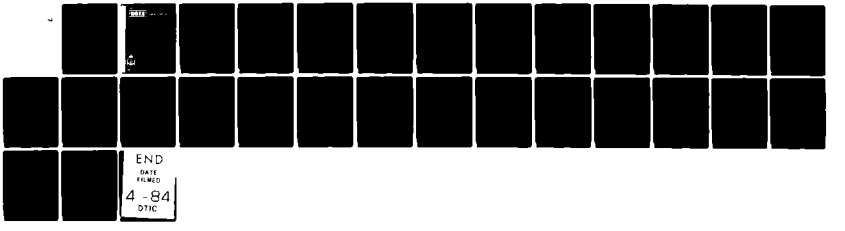

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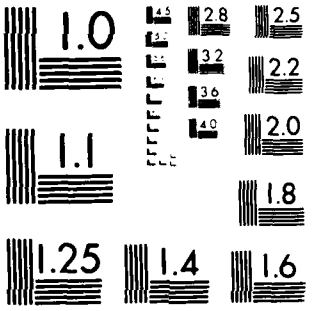

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SUFFIELD REPORT

NO. 378

IMMUNOCHEMICAL IDENTIFICATION OF NEWCASTLE DISEASE VIRUS STRUCTURAL PROTEINS (U)

AD A138830

by

R.E. Fulton and R. Shew[1]

Project No. 16B10

January 1984

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ABSTRACT

The immunochemical reactivity of the structural polypeptides of two strains of Newcastle Disease Virus (La Sota and B_1), when reacted with hyper-immune polyclonal antisera, has been studied. Methods are described for the separation of NDV proteins by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (reduced and non-reduced), the electrophoretic transfer of separated proteins to nitrocellulose paper, and the subsequent identification of NDV antigenic determinants by a sensitive enzyme-linked immunosorbent assay. Using these methods, a number of new, minor polypeptides have been detected which hitherto have not been described.

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INTRODUCTION

Newcastle disease virus (NDV) is a paramyxovirus consisting of a strand of ribonucleic acid (RNA) surrounded by protein to form a helical nucleocapsid which, in turn, is enclosed within a lipoprotein membrane of host origin. The virus contains eight virus-coded structural polypeptides ranging in molecular weight from 13,000 to 150,000 daltons (5, 7, 15, 16, 18). One of the most notable properties of NDV is the wide variation in ability of different strains to cause disease and death in chickens. A spectrum of virulence between the extremes of very pathogenic (death within three days of infection) and non-pathogenic (no disease) has been reported (1). In spite of this considerable variation in virulence, strains with widely

differing biological properties are not distinguishable using conventional serological procedures. In addition, in a study of 14 different strains, Moore et al. (15) failed to show any differences in structural polypeptides between strains when analyzed, under reduced conditions, by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Although one of the polypeptides of several of the strains ran under non-reduced conditions as a dimer, no correlation to virulence could be found. More recently, experimental evidence has been reported which supports the concept that susceptibility of the envelope glycoproteins to proteolytic cleavage is of prime importance in determining pathogenicity and that this susceptibility is a structural property of the virus (11).

This present work was undertaken to determine whether polyclonal antibodies could be used to detect differences in immunological reactivity of the structural polypeptides of different strains of NDV. The immunochemical reactivity of the structural polypeptides of two relatively non-pathogenic strains of NDV (B_1 and La Sota), when reacted with hyperimmune polyclonal antiserum, have been studied. We describe methods for the separation of NDV proteins by SDS-PAGE (reduced and non-reduced), the electrophoretic transfer of separated proteins to nitrocellulose paper (Electroblot), and the subsequent identification of NDV antigenic determinants by a sensitive enzyme-linked immunosorbent assay (ELISA).

We have identified in our NDV samples the same major polypeptides as have been reported by others (5, 7, 15, 16, 18). In addition, by use of a sensitive silver stain for protein, the presence of additional minor polypeptides not previously described was detected. Many of the NDV proteins, including the new, minor polypeptides, react with NDV antibody.

MATERIALS AND METHODS

Virus

NDV vaccine strains, B_1 and La Sota, originally obtained from Salisbury Laboratories (Kitchener, Ontario) were passaged and grown to volume in the allantoic sac of 10-day-old embryonated hens' eggs. Virus from harvested allantoic fluid was purified by differential centrifugation followed by a series of discontinuous and continuous sucrose gradient centrifugation steps.

Allantoic Fluid

Allantoic fluid from uninfected 10 day-old embryonated hens' eggs was lyophilized and stored at 4°C until use. Prior to PAGE, the lyophilized fluid was resuspended in distilled water and incubated at 37°C for 3 h. Undissolved material was pelleted in an Eppendorf Model 5412 desktop centrifuge and the supernate analyzed by SDS-PAGE.

SDS-PAGE

SDS-PAGE was performed with 9% and 11% gels and the buffer system of Laemmli (12). After dialysis against Tris-HCl buffer (62.5 mM Tris-HCl, pH 6.8), aliquots of each virus and of control allantoic fluid, containing approximately 20 µg of protein each, were loaded onto SDS polyacrylamide vertical slab gels. Both reduced and non-reduced samples of each virus and of control allantoic fluid were electrophoresed simultaneously. Molecular weight standards were included with each electrophoretic run.

To obtain optimal separations and band resolution, the porosity, pH and Tris and glycine concentrations in the stacking and separating gels were investigated. The following procedures and conditions were those found to

yield optimal results. Gels containing 6% (stacking gel), 9% and 11% acrylamide were prepared from a monomer stock solution of 30% (w/v) acrylamide (Biorad Laboratories, Mississauga, Ontario) and 0.8% (w/v) N,N-methylene-bis-acrylamide (Biorad Laboratories). The gels were polymerized chemically by the addition of 0.025% (v/v) tetamethyl-ethylenediamine (TEMED) (Sigma Chemical Company, St. Louis, Missouri) and ammonium persulfate (Biorad). Gels of 1.5 mm thickness, 16 cm width and 12.5 cm length were prepared using glass plates as a mould. The stacking gels were 1 cm in length and contained 0.0625 M Tris-HCl (pH 6.8) and 0.1% SDS (Biorad Laboratories). The separating gel contained 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS. The electrode buffer (pH 8.3) consisted of 0.025 M Tris-HCl, 0.1824 M glycine (Sigma Chemical Company) and 0.1% SDS. Non-reduced samples (22-65 µl volumes) contained (in final concentration) 0.0625 M Tris-HCl (pH 6.8), 0.1% SDS (2.0% SDS for high and low molecular weight standard preparations (Sigma Chemical Company)), 10% glycerol and 10 µl of 0.1% bromophenol blue (Biorad Laboratories) as the tracking dye. Reduced samples contained the same ingredients but with the addition of 1% β-mercaptoethanol (Eastman Chemical International, Don Mills, Ontario). Reduced samples were further denatured by immersion into boiling water for 5 min (to insure complete dissociation of the proteins). Electrophoresis was carried out at 4°C, using a current of 15 mA/gel, until the bromsphenol blue marker entered the separating gel (about 1 h), at which time the current was increased to 30 mA/gel until the bromophenol blue marker reached the bottom of the gel (about 3 h). A Biorad Protean dual slab cell was used for all gel electrophoreses.

Staining of Gels

a) Coomassie Brilliant Blue/Silver

Upon completion of electrophoresis, gels were stained for 1 h in a solution of Coomassie brilliant blue R250 (Sigma Chemical Company) (0.125%

Coomassie brilliant blue, 9.2% acetic acid and 50% methanol). Gels were destained in several changes of hot 40% methanol/10% acetic acid, until the background was clear, and then incubated in two changes of 10% ethanol/5% acetic acid (v/v) for a total of 1 h at room temperature. The gels were then stained with silver (silver staining kit, Biorad Laboratories) according to the instructions given by the supplier. Briefly, the gels were rinsed with triple distilled water and immersed for 10 min in oxidizing solution, followed by extensive washing in triple distilled water until all excess oxidizer was removed (indicated by colorless background). The gels were then immersed for 30 min at room temperature in silver reagent followed by a 10 min wash in triple distilled water. Gels were then incubated in two to three changes of developer until the desired band intensity was reached. Development was stopped with 5% (v/v) acetic acid.

b) Periodic Acid-Schiff (PAS)

Gels were fixed and stained with PAS reagent according to Segrest and Jackson (19). Upon completion of electrophoresis the gels were incubated overnight in fixative (40% ethanol, 50% acetic acid), treated with 1% periodic acid (in 7% acetic acid) for 2 h, then decolorized in 1% sodium bisulfite (in 0.1 N HCl) for 1 h. Several changes of sodium bisulfite were required to decolorize the gels completely. The gels were then rinsed with distilled water and incubated in Schiff's reagent (Sigma Chemical Company) for 24 h at room temperature. Pink coloration indicated positively stained glycoproteins.

c) Ethidium Bromide

To test for the presence of virus RNA in separated polypeptides, gels were stained with ethidium bromide. The following procedure (4, 9) was adopted. Upon completion of electrophoresis, the gels were incubated in the

dark at room temperature for 1 h in a solution of ethidium bromide (Sigma Chemical Company) (4 µg/ml aqueous stock diluted approximately 1/5000 in distilled water just prior to use). Bands were visualized under long-wave UV (366 nm).

d) Methoxyphenyl Neuraminic Acid (MPN)

The synthetic substrate of Tuppy and Palese (21), 2-(3'-methoxyphenyl)-N-acetyl-α-neuraminic acid (MPN), was used to test for the presence of neuraminidase activity on electrophoresed gels. MPN is a chromogenic substrate which, when incubated with neuraminidase in the presence of diazonium salt, produces a red precipitate at the site of activity.

To remove residual SDS, gels were fixed overnight at room temperature in 40% methanol/10% acetic acid. Following the procedure of Bucher et al. (6), gels were then incubated in 0.1 M sodium phosphate buffer, pH 6.6, for 30 min at room temperature. Following a brief rinse in distilled water, the gels were then incubated for 30 min at room temperature in a mixture of 10 mg MPN (National Institute of Allergy and Infectious Diseases, Bethesda, Maryland) and 10 mg of the diazonium salt of 4-amino-2,5-dimethoxy-4'-nitroazobenzene (Black K Salt) (ICN Pharmaceuticals, Plainview, New York) in 5.0 ml of 0.2 M sodium phosphate buffer containing 0.1 M $CaCl_2$, pH 6.6. The gels were placed in 7% acetic acid for storage.

Electroblot

Proteins to be assayed for antigenic activity were transferred from the gels to nitrocellulose paper by electroblotting, a procedure originally described by Renart et al. (17). Immediately following electrophoresis, the gel was rinsed with distilled water and placed on a sheet of wetted blot

adsorbent filter paper. A pre-wetted[1] sheet of nitrocellulose paper was then laid on top of the gel, and carefully rubbed to ensure a nearly adhesive contact between the membrane and the gel. A second sheet of wetted blot adsorbent filter paper was then placed on top of the membrane and the sandwich held together between sponge pads and placed in electroblot buffer consisting of 25 mM Tris-HCl (pH 8.3), 192 mM glycine/20% (v/v) methanol, and 0.1% SDS. Proteins were electroblotted for 18 h at room temperature, at a current of 40 v in an uncooled trans-blot cell (Biorad Laboratories). The buffer was stirred throughout. Upon completion of electroblotting, the membrane or portions thereof were either stained immediately with Coomassie blue, stained immediately by ELISA, or were air-dried and stored at 4°C for ELISA staining at a later date.

ELISA

a) Determination of Optimum Reagent Concentrations

To evaluate chloronapthol as a developer for ELISA and to determine optimum reagent concentrations to be used in detection of NDV electroblotted polypeptides, a dot-immunobinding assay (10) was performed in a box-type titration using purified B_1 strain of NDV whole virus of known protein concentration (1.35 µg/ul) as antigen. Two-fold dilutions (undiluted to 1/256) of virus were prepared and, with the exception of the undiluted sample, 2.5 µl of each was spotted directly onto nitrocellulose paper (5 µl of undiluted sample was applied). The spotted proteins were then dried onto the membrane by overnight incubation at 37°C. The nitrocellulose paper was cut with a scalpel into 4 mm squares, each bearing a spotted virus dilution and each square was placed, protein side up, into an appropriate well of a

[1] The nitrocellulose paper was touched to the buffer and allowed to wet by capillary action, thus minimizing entrapment of air bubbles in the matrix.

96-well round bottom, Cooke^{R} microtiter plate. To block unoccupied protein binding sites on the paper, 150 µl of 3% (w/v) bovine serum albumin (BSA) in Tris-buffered saline (TBS) (10 mM Tris-HCl, 0.9% NaCl, pH 7.4) (BSA/TBS) was added to each well and the plate incubated for 4 h at room temperature. The blocking solution was then aspirated off with a Pasteur pipet connected to a suction line. Two-fold dilutions (1/200 to 1/3200) of primary antibody (guinea pig anti-NDV IgG[1], 6 mg/ml) were prepared in BSA/TBS and 100 µl of each added to appropriate wells of the microtitre plate. After incubation for 4 h at room temperature, the primary antibody was aspirated off and each membrane washed with several changes of TBS. One hundred microliters of peroxidase-labelled anti-guinea pig IgG (Miles Laboratories, Rexdale, Ontario), diluted 1/100 in BSA/TBS, was then added to the well and incubated for 3 h at room temperature. The membranes were washed for 15 min with several changes of TBS, after which they were developed in 4-chloro-1-naphthol (Sigma Chemical Company) and hydrogen peroxide. Chloronaphthol was prepared as a 3 mg/ml stock solution in methanol and stored at 4°C in the dark. Just prior to use, it was diluted with five volumes of TBS and hydrogen peroxide was added to a final concentration of 0.01% immediately before addition to the wells. One hundred and fifty microliters of developing solution was added to each membrane-containing well. A positive reaction was observed as a paper-bound colored (dark blue) precipitate.

In preliminary trials, o-dianisidine dihydrochloride (Sigma Chemical Company) was used as developer (20), as follows. Immediately prior to use, 450 µl of 30% hydrogen peroxide was added to a freshly prepared solution of dianisidine (20 mg o-dianisidine dihydrochloride + 37.2 mg ethylenediaminetetraacetic acid (EDTA) dissolved in 80 ml acetate buffer. pH 3.8) and the volume adjusted to 100 ml with acetate buffer. Use of this developer was

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[1] Produced in house and immunopurified by Jackson ImmunoResearch Laboratories, Avondale, Pennsylvania.

terminated when it was observed that the color reaction occurred in the solution surrounding the paper-bound antigen-antibody complex rather than being precipitated on the paper.

b) Detection and Identification of Electroblotted Proteins

The electroblotted nitrocellulose paper was cut longitudinally into strips, each corresponding to an individual sample lane, and each strip placed in a separate trough of a specially constructed teflon staining tray. Primary antibody was used at a dilution of 1/2000. Peroxidase-labelled secondary antibody was diluted 1/500. All other conditions and reagents were as described for the dot immunobinding assay, above.

Protein Assay

Protein concentration of purified preparations of B_1 and La Sota strains of NDV and of control allantoic fluid were determined by the Biorad protein assay (Biorad Laboratories).

Molecular Weight Standards

High (H) and low (L) molecular weight (MW) standard protein mixtures were purchased from Biorad Laboratories (HMW:40,000-250,000; LMW:10,000-100,000) and Sigma Chemical Company (HMW:29,000-205,000; LMW:14,200-66,000).

RESULTS

The major virus protein bands revealed by Coomassie blue staining were of the following approximate molecular weights >200,000, 75,000, 66,000, 56,000, 49,000, 41,000, 32,000 and 13,000. Using the more sensitive silver

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stain, a multitude of additional minor bands, most of which were of molecular weight <41,000, were resolved. For each virus strain the electrophoretic profiles of reduced and non-reduced virus were very similar. Minor differences, however, were observed. For example, the protein band at MW 75,000 was straight and flat in the reduced samples but in the non-reduced, appeared to "smile" upwards. No differences in electrophoretic profile between B_1 and La Sota strains could be recognized in gels electrophoresed under either reduced or non-reduced conditions. Higher molecular weight proteins were separated with better resolution on 9% gels, whereas lower molecular weight proteins were better separated on 11% gels. Hence, both 9% and 11% gels were routinely used.

Glycoproteins, detected by periodic acid-Schiff staining, were identified at three positions on the gel, corresponding to molecular weights of 75,000, 56,000 and 13,000. No RNA was detectable by ethidium bromide staining in either the reduced or non-reduced NDV polypeptides. Similarily, neuraminidase activity was not detectable by MPN in any of the separated proteins.

As visualized by Coomassie blue staining, non-reduced control allantoic fluid separated into four bands corresponding to molecular weights of approximately 95,000, 56,000, 32,000 and 30,000. In reduced samples two bands, one major one at 65,000-70,000 and another minor one at a molecular weight of approximately 40,000 were observed. The protein band observed at 56,000 in non-reduced gels was of particular interest since a protein of similar molecular weight had been observed in NDV samples.

Heavy staining with Coomassie blue was observed at the origins of the stacking and separating gels in all tracks containing virus. This material was PAS positive but did not stain for RNA with ethidium bromide. This phenomenon was not observed in tracks containing control allantoic fluid or

molecular weight standards.

An electroblotting period of 18 h was found to be required to achieve a quantitative transfer of all SDS-PAGE separated polypeptides to nitrocellulose paper. The efficiency of transfer of high molecular weight proteins was improved by the addition of 0.1% SDS to the electroblot buffer, and by using overnight runs. That all protein had been transferred from the gel to the paper was inferred from the finding that electroblotted gels, when stained with silver, contained no visible bands. Coomassie blue and silver stain were evaluated as general protein stains for direct staining of electroblotted nitrocellulose paper, but were found to be inadequate. Intense nonspecific background staining was observed on Coomassie blue stained papers and the silver stain, although marginally better, was disadvantaged by the finding that bands appeared negatively stained against a pale yellow background. The presence, therefore, of NDV proteins on the electroblotted paper could only be demonstrated by a positive ELISA reaction.

The dot-immunobinding assay box titration was designed to determine conditions for detection of the lowest and highest concentrations of NDV antigens to be expected on electroblotted nitrocellulose papers. Since a 20 µg virus sample routinely loaded on the gel separated into eight polypeptides detectable by Coomassie blue, each polypeptide detected by this method was estimated to contain approximately 2.5 µg of protein. Minor polypeptides detectable by the silver stain were estimated to be in a concentration range of at least 0.05-0.01 µg (the limits of sensitivity of this stain) (2). Hence, virus dilutions of purified B_1 of undiluted to 1/256, representing protein concentrations ranging from 6.735 to 0.0133 µg, were considered to adequately cover the range of concentrations of protein which could, theoretically, be transferred to and detected on nitrocellulose paper. Primary antibody was used in dilutions which, for most concentrations of antigen, would be in excess (1/200-1/3200, corresponding to IgG concentra-

tions ranging from 3 µg to 0.1875 µg). Similarly, the peroxidase-conjugated secondary antibody was used in excess (1/100). Results of the dot-immunobinding assay indicated that, even at the highest dilution of virus antigen (corresponding to 13.3 ng protein) and the highest dilution of primary antibody, an endpoint in the titration was not reached. The color intensity of the ELISA reaction was equally as intense for the most dilute concentrations of antigen and NDV antibody as it was for the most concentrated. Controls consisting of nitrocellulose paper alone, TBS alone, and NDV antigen without peroxidase-labelled secondary antibody (all with color reagent), exhibited no interferring background staining. A primary antibody dilution of 1/3200 was, therefore, considered to be appropriately in excess and was used in all subsequent ELISA.

Using the same conditions as those established for the dot immuno-binding assay, ELISA performed on electroblotted gels of SDS-PAGE separated virus polypeptides revealed multiple reaction bands corresponding not only to all of the major proteins as visualized by Coomassie blue staining, but also to many of the small molecular weight polypeptides (<41,000) seen only after silver staining. Positive ELISA reactions were observed with both reduced and non-reduced virus samples, although there was no detectable difference in band profile between strains. Noteable also was intense staining at the origins of the separating and stacking gels. ELISA performed on electro-blotted gels of allantoic fluid polypeptides was negative, indicating non-specificity of anti-NDV IgG for allantoic fluid antigens. Hence the 65,000 dalton NDV polypeptide and the 65,000 dalton allantoic fluid polypeptide with which it comigrated, were shown to be immunologically distinct.

DISCUSSION

NDV, both in the reduced and in the non-reduced forms, separated into eight polypeptides (detectable by Coomassie blue staining), ranging in

molecular weight from >200,000 for the largest protein to 13,000 for the smallest protein. These findings are in close agreement with those previously reported for NDV (5, 7, 15, 16, 18), and as such, each polypeptide at its respective molecular weight may be assigned with the following morphological/biochemical identities: >200,000: large protein (L); 75,000: hemagglutinin and neuraminidase glycoproteins (HN); 66,000: fusion glycoprotein (F_0); 56,000: fusion and hemolysis glycoprotein (F); 49,000: nucleocapsid protein (NP); 41,000: cleavage product protein (C); 32,000: membrane protein (M); 13,000: small protein (S). In addition to these major polypeptides, minor polypeptides, most of which were of molecular weight <41,000, were identified by the more sensitive silver stain. That these polypeptides were virus-specific was attested to by the fact that most reacted with antibody to NDV on transblot. These virus-specific proteins have not been previously described and their morphological/biochemical identities are therefore unknown. It is possible that they represent breakdown products of the larger polypeptides identified by Coomassie blue, or they may be unique proteins. Radiolabelling experiments, utilizing pulse-chase techniques, might be useful in elucidating the origin and identity of these small proteins.

The separated polypeptide PAGE patterns of reduced and non-reduced NDV proteins were very similiar. This suggests that very little disulfide bridging occurs between peptides and possibly, that there are no disulfide-linked dimers or polymers of individual polypeptides. Further information concerning the polypeptide structure of NDV might be obtained by running the virus on non-SDS gel systems, or by utilizing different conditions or reagents, e.g. high salt concentrations or enzymes, respectively, to disrupt the native structure of the whole virus.

Because of the wide range in size of polypeptides comprising NDV, all of the polypeptides could not be adequately separated on one gel. In order

to resolve the major low molecular weight proteins (≤13,000), small pore gels were required, with the result that the major large proteins (MW>200,000) did not enter the gel. Conversely, to resolve the high molecular weight proteins, large pore gels were required and the low molecular weight proteins either ran at the tracking dye front, or were electrophoresed off the end of the gel. Hence, in order to maximize resolution of all component proteins, it was necessary to routinely and simultaneously run two different gel porosites (9% and 11%).

Glycoproteins were identified by PAS staining at three positions on the gel, corresponding to molecular weights of approximately 75,000, 56,000 and 13,000. These PAS-positive bands correspond to those which have been previously described as HN, F and S proteins, all of which are believed to be glycoproteins (5, 7, 15, 16, 18). One other polypeptide which is thought to be a glycoprotein, (F_0), did not stain by the PAS method on our gels. The reason for this discrepancy is not clear.

In spite of the fact that the hemagglutinin and neuramindase activities of NDV are known to reside in the HN glycopeptide (MW 75,000) (5, 7, 15, 16, 18), using the MPN staining procedure we were unable to demonstrate any enzyme activity in this glycoprotein on our gels. This might be accounted for by the fact that the substrate used (MPN) had been stored for a long period of time (four years) and may have thus degraded; the product is known to undergo auto-hydrolysis if exposed to humidity. Alternatively, it is quite possible, if not probable, that enzyme activity is lost as a result of denaturation with heat and disruption with SDS. The fact that the neuramindase of A_2 strains of influenza virus is active after such treatment (6, 13) is indeed remarkable (6). There is no reason to suppose that the neuraminidase of NDV is equally stable.

Ethidium bromide staining failed to detect the presence of viral

nucleic acid in gels after electrophoresis. A number of factors may have contributed to this finding. Primary among these is the probability that viral nucleic acid did not enter the gels. The genome of NDV is known to consist of a single strand of RNA with molecular weight of 5.1×10^6 - 5.7×10^6 (7). The molecular size of this molecule, therefore, would exclude the possibility of its penetration into a 9% or 11% resolving gel, or even into a 6% stacking gel. Even if the molecule could be resolved in these gels, one would not expect to find it in association with any of the viral polypeptides, since SDS is known to eliminate the interaction of proteins and nucleic acids (14). The staining procedure used in our experiments is one which is more commonly used to reveal the presence of double-stranded nucleic acid in agarose gels, usually of quite low porosity (0.5-1.0%). In order to stain single-stranded nucleic acid, it is necessary to carry out the staining procedure in the presence of $MgSO_4$ (8). In addition, in order to penetrate highly cross-linked gels, as used in our experiments, it is likely that a much higher concentration of ethidium bromide and/or longer staining time would be required. In addition, inclusion of a control gel, consisting of a known concentration of electrophoresed single-stranded RNA, would be helpful in assessing the validity of the test procedure.

Regardless of the denaturation and dissociation system used, i.e. dissociation using SDS alone or dissociation and denaturation with SDS, boiling, and reduction with β-mercaptoethanol, heavy staining by Coomassie blue was noted to occur at the origins of both the stacking and the resolving gels, indicating the presence in these regions of protein of >220,000 molecular weight. This material was found to be PAS positive and therefore to consist of glycoprotein. In addition, by ELISA on electroblotted nitrocellulose paper, it reacted specifically with NDV antibody, hence indicating its viral origin. This material might consist of whole or partial virus fragments which were not completely dissociated and denatured, or, could conceivably be very large as yet undescribed polypeptides which were too

large to enter the gel under the conditions used. Being PAS positive, it is most likely that these are membrane components of the virus containing the HN glycoprotein. More vigorous dissociation and/or reduction procedures may be necessary to effect complete dissociation of this virus. It might also be informative to determine whether this material is resolvable on high porosity gels, e.g., 0.5-2% agarose, or 2-3% polyacrylamide.

Both Coomassie blue and the silver stain proved to be unsuitable as general protein stains on nitrocellulose paper. The use of amido black for this purpose has been described (3) and should be evaluated as an alternative approach.

The dot-immunoblot ELISA was used to evaluate 4-chloro-1-napthol as an ELISA developer and to determine optimum reagent conditions for detection of NDV electroblotted polypeptides. Chloronapthol proved to be a very sensitive color reagent and was far superior to o-dianisidine dihydrochloride for ELISA procedures on nitrocellulose. Use of the latter color reagent resulted in color reactions which developed in the solution surrounding the antigen-antibody complex and which quickly diffused into the surrounding media. The color reaction produced by chloronapthol, however, occurred in situ and the colored product was precipitated directly onto the nitrocellulose paper at the site of antigen-antibody fixation. Papers developed in this manner have been preserved for weeks without loss of stain intensity. The dot-immunoblot assay is an extremely sensitive procedure for detection and identification of antigens applied directly to nitrocellulose paper. In our experiments, as little as 13.3 ng of virus protein was detected. Since an endpoint in antigen titration was not reached in our assays, one can assume that the limit of detection sensitivity of this procedure exceeds 13.3 ng protein. Such a procedure might prove useful for rapid identification of viruses in patients' specimens or in aerosol samples and the feasibility of this approach should be investigated.

It is of interest to note that a large number of NDV polypeptides reacted in the ELISA, in spite of the fact that the virus protein had been dissociated and denatured by treatment with detergent, high temperature and reducing agent. Such treatment destroys the three dimensional configuration of the antigenic determinants and the native conformationally functional state of the protein is thereby lost. The fact that NDV antibody is able to recognize specific antigenic determinants suggests that antibody specificity for these NDV antigens is sequence dependant rather than conformation dependent.

Although the electroblot ELISA using polyclonal antibodies to the LaSota strain of NDV failed to detect strain differences between the structural polypeptides of electroblotted proteins of B_1 and LaSota, monoclonal antibodies may be useful in this regard. A monoclonal antibody which could identify strain specific antigenic determinants would not only generate new information on the structural basis of strain differentiation, but could also be of important practical significance in distinguishing between pathotypes. Further studies investigating the immunochemical reactivity of monoclonal antibodies to the LaSota strain of NDV, when reacted with the structural polypeptides of a number of different strains of NDV, are currently underway.

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APPENDIX

A. Tris Running Buffer (Electrode Buffer)

0.025 M Tris-HCl (0.1824 M Gly (pH 8.3), 0.1% SDS)

1. 4x concentrated stock: 24.22 g Tris + 109.6 g Gly made up to 2.0 L with distilled water, pH 8.3. Store at 4°C.

2. 1x buffer: 1.0 L of 4x Tris (stock) + 2960 ml of distilled water + 40 ml of 10% SDS

B. Upper (Stacking) Gel Buffer

Make as a 4x concentrated stock: 3.028 g Tris, 0.4 g SDS made up to 100 ml with distilled water, pH 6.8. Store at room temperature. Degas.

C. Lower (Separating) Gel Buffer

Make as a 4x concentrated stock: 18.17 g Tris + 0.4 g SDS made up to 100 ml with distilled water, pH 8.8. Store at room temperature. Degas.

D. 30% Acrylamide, 0.8% Bis-Acrylamide

30 g acrylamide + 0.8 g methylene-bis-acrylamide made up to 100 ml with distilled water. Filter, degas and store in the dark at 4°C.

E. 2% Ammonium Persulfate

Make fresh daily: 0.1 g ammonium persulfate + 5 ml distilled water.

F. Sample Buffer

1. Make Tris buffer: 6.06 g Tris + 0.4 g SDS made up to 100 ml with distilled water, pH 6.8.

2. 10 ml glycerol + 30 ml of 10% SDS + 12.5 ml Tris buffer + 47.5 ml distilled water

3. For B1 and La Sota samples, add an equal volume of sample buffer.

4. Degas. Store at room temperature.

G. 10% SDS

20 g SDS made up to 200 ml with distilled water. Degas. Store at room temperature.

H. Tracking Dye

1. Make as a 0.5% aqueous stock: 0.05 g bromophenol blue made up to 10 ml with distilled water. Stir until no more dissolution. Store at room temperature.

2. Prior to use, dilute with nine volumes of sample buffer (see F, above).

3. Use 10 µl for each sample aliquot, ie. 10 µl/lane on gel.

I. Coomassie Brilliant Blue (R250) Stain

1. 1.25 g Coomassie blue + 92 ml acetic acid + 500 ml methanol + distilled water up to 1 L

2. Filter and store at room temperature.

J. Destain (for Coomassie Brilliant Blue Stain)

1. 500 ml distilled water + 400 ml methanol + 100 ml acetic acid

2. Store at room temperature.

K. Gels (for Protean cell); 1.5 mm x 16 cm x 12.5 cm

(Each recipe for 11% and 9% gels makes one slab gel)

	11% (ml)	9% (ml)	6% (for two gels) (ml)
distilled water	12.88	15.217	10.744
4x Tris buffer	8.75 (C, above)	8.75 (C, above)	5.0 (B, above)
30% acrylamide/0.8% bis	12.838	10.5	4.0
2% ammonium persulfate	0.525	0.525	0.25
TEMED	0.0084	0.0084	0.006

NOTE: ALL REAGENTS: DEGASSED, ROOM TEMPERATURE, STIRRED THOROUGHLY BY SWIRLING.

L. Silver Stain

All silver stain reagents are prepared according to the Biorad Silver Stain Kit.

M. PAS Stain

1. 1% periodic acid: 1.0 g periodate made up to 100 ml with 7% acetic acid

2. 1% sodium bisulfite: 1.0 g sodium bisulfite up to 100 ml with 0.1 N HCl.

N. Electroblot Buffer
(25 mM Tris-HCl (pH 8.3), 192 mM Glycine)/20% Methanol (v/v), 0.1% SDS,

1. Dissolve 12.11 g Tris in 2 L of distilled water and adjust pH to 8.3.

2. Add 57.68 g glycine. Dissolve.

3. Add 5.0 g SDS. Dissolve.

4. Adjust volume to 4 L with distilled water. Mix.

5. Add 1 L methanol. Mix.

O. 3% BSA

1. 6.0 g BSA made up to 200 ml with Tris-buffered saline

P. Tris-buffered Saline
(10 mM Tris-HCl (pH 7.4), 0.9% NaCl)

1. 1.211 g Tris made up to 3.5 L with distilled water. Adjust pH to 7.4.

2. Add 36 g NaCl. Dissolve.

3. Adjust volume to 4 L with distilled water.

Q. Chloronaphthol

1. Stock: 150 mg 4-chloro-1-naphthol made up to 50 ml with methanol. Refrigerate in the dark at 4°C.

2. 5 ml chloronaphthol stock + 25 ml Tris-buffered saline (P, above).

3. Add 10 µl of 30% H_2O_2. Mix.

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DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall document is classified)

1 ORIGINATING ACTIVITY

DRES

2a. DOCUMENT SECURITY CLASSIFICATION

Unclassified

2b. GROUP

3 DOCUMENT TITLE

IMMUNOCHEMICAL IDENTIFICATION OF NEWCASTLE DISEASE VIRUS STRUCTURAL PROTEINS

4 DESCRIPTIVE NOTES (Type of report and inclusive dates)

5. AUTHOR(S) (Last name, first name, middle initial)

R.E. Fulton and R. Shew

6 DOCUMENT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
January 1984	25	21

8a. PROJECT OR GRANT NO.	9a. ORIGINATOR'S DOCUMENT NUMBER(S)
16B10	SR 378
8b CONTRACT NO.	9b. OTHER DOCUMENT NO.(S) (Any other numbers that may be assigned this document)

10. DISTRIBUTION STATEMENT

Unlimited

11 SUPPLEMENTARY NOTES	12. SPONSORING ACTIVITY

13. ABSTRACT

The immunochemical reactivity of the structural polypeptides of two strains of Newcastle Disease Virus (La Sota and B_1), when reacted with hyper-immune polyclonal antisera, has been studied. Methods are described for the separation of NDV proteins by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (reduced and non-reduced), the electrophoretic transfer of separated proteins to nitrocellulose paper, and the subsequent identification of NDV antigenic determinants by a sensitive enzyme-linked immunosorbent assay. Using these methods, a number of new, minor polypeptides have been detected which hitherto have not been described.

KEY WORDS

Newcastle Disease Virus, NDV, structural polypeptides, SDS-gel electrophoresis, electroblot, ELISA, dot-blot

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